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New Pregnane Glycosides Isolated from *Caralluma hexagona* Lavranos as Inhibitors of α-Glucosidase, Pancreatic Lipase, and Advanced Glycation End Products Formation

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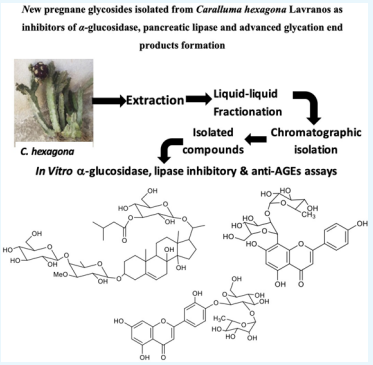
ABSTRACT: *Caralluma hexagona* Lavranos (Family Asclepiadaceae) is an endemic herb in Yemen and Saudi Arabia, traditionally used to treat diabetes, abdominal pain, and stomach ulcers. Different extracts, fractions, and main constituents of *C. hexagona* were evaluated for their inhibitory activity against key enzymes in diabetes and hyperlipidemia, i.e., α-glucosidase and pancreatic lipase. In addition, the antioxidative effect and inhibition of advanced glycation end products (AGEs) were also assayed. Using a bioguided approach, the crude aqueous, methanolic extracts, methylene chloride (CH_2Cl_2), Diaion HP20 50% MeOH (DCF-1), and 100% MeOH (DCF-2) fractions of *C. hexagona* were evaluated for their possible α-glucosidase and pancreatic lipase inhibition and antioxidant activity. In addition, inhibition of AGE generation using bovine serum albumin (BSA)-fructose, BSA-methylglyoxal, and arginine-methylglyoxal models was carried out. Moreover, the main constituents of the most active fraction were isolated and identified using different chromatographic and sprectroscopic methods. From the most active CH_2Cl_2 fraction, four new pregnane glycosides were isolated and identified as 12β-*O*-benzoyl 3β,8β,12β,14β,20-pentahydroxy-(20*S*)-pregn-5-ene-3-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-digitaloside (**1**), 3β,8β,14β,20-tetrahydroxy-(20*S*)-pregn-5-ene-3-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-digitaloside-20-*O*-3-isoval-β-D-glucopyranoside (**2**), 3β,8β,14β,20-tetrahydroxy-(20*R*)-pregn-5-ene-3-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-digitaloside-20-*O*-3-isoval-4-benzoyl-β-D-glucopyranoside (**3A**), and 3β,8β,14β,20-tetrahydroxy-(20*R*)-pregn-5-ene-3-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-digitaloside-20-*O*-3,4 di-benzoyl-β-D-glucopyranoside (**3B**). Among the tested samples, the highest trolox equivalent (TE) antioxidant capacity (TEAC) was observed for DCF-1 with values of 128.53 ± 5.07, 378.58 ± 5.19, and 106.71 ± 5.66 μM TE/mg using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant potential (FRAP) assays, respectively. The isolated apigenin-8-*C*-neohesperoside showed the highest antioxidant capacity (168.80 ± 1.80 and 278.21 ± 13.26 μM TE/mM) in DPPH and FRAP, respectively, while luteolin 4′-*O*-β-D-neohesperidoside had the highest TEAC (599.19 ± 9.57 μM TE/mM) in ABTS assay. Compounds **1**, **2**, and the mixture **3A** and **3B** inhibited α-glucosidase with IC_{50} values of 0.92 ± 0.02, 0.67 ± 0.01, and 0.74 ± 0.02 mM, respectively. In the AGE assays, DCF-1 showed the highest inhibitory effect in BSA-fructose and arginine-methylglyoxal models with IC_{50} values of 0.39 ± 0.02 and 0.77 ± 0.10 mg/mL, respectively. Among the isolated compounds, flavonoid compounds showed the highest antiglycation effect, while pregnanes revealed higher α-glucosidase inhibition. In conclusion, the current study revealed that *C. hexagona* is a promising Yemeni natural remedy, of which the major content of pregnane glycosides and flavonoids could be considered as a new therapeutic candidate targeting the metabolic syndrome.



1. INTRODUCTION

The metabolic syndrome gathers symptoms such as abdominal obesity, hypertension, dyslipidemia, and disturbance of glucose metabolism (insulin resistance, high blood glucose, and impaired glucose tolerance).[1,2] Diets containing a high level of carbohydrates are partially responsible for diabetes, but obesity is also commonly considered as a risk factor for type 2 diabetes mellitus, which emphasizes the probability of lipid effects mediating this process as well.[3]

Advanced glycation end products (AGEs) are non-enzymatic modifications of proteins, lipids, or nucleic acids after their reaction with the aldehyde group of reducing sugars (e.g., fructose and glucose). In this field, the most common reactive molecules are dicarbonyl compounds such as glyoxal and methylglyoxal.[4] The glycation of protein occurs as a non-enzymatic *in vitro* reaction between the sugar's aldehydic

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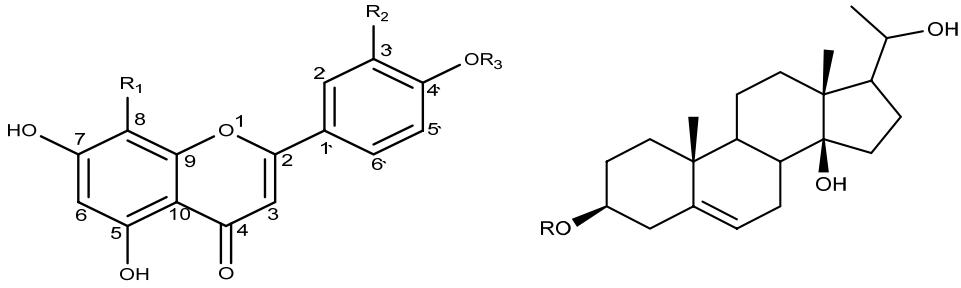

7: R_1= **H.** R_2= **OH.** R_3= **Rha-(1→2)-Glc.**

8: R_1= **Rha-(1→2)-Glc.** R_2= **H** R_3= **H.**

9: R= H.

10: R= Glc.

Figure 1. Structures of the isolated compounds of *C. hexagona*.

moiety and the amino moiety of proteins producing Schiff bases, called the Maillard reaction. Then, they undergo Amadori rearrangement, resulting in stable compounds named Amadori products. When exposed to oxidation or low values of pH, these products are decomposed to produce reactive carbonyls, including methylglyoxal.[5−7] AGEs can be exogenously obtained through food or endogenously.

Long-term hyperglycemia is a critical cause for the increase of oxidative stress and the production of AGEs,[8] which consequently build up in the body. Thus, the AGE accumulation plays an essential role in critical diseases such as diabetes, obesity, and atherosclerosis.[4,9] The complications related to metabolic syndrome could be alleviated by controlling postprandial hyperglycemia, fat absorption, and AGE formation through inhibition of the enzymes responsible for the hydrolysis of glycosides and lipids, as well as non-enzymatic glycation. In the past 10 years, research on the pharmacological activities of *Caralluma* sp. has shown that most of these plant species could be possible sources of new active compounds.[10−17] In addition to other metabolites such as flavonoid glycosides, pregnane glycosides were reported to be the most characteristic constituents of this genus. Several recent studies highlight their importance as medicinal plants to fight against particular diabetes, obesity, gastrointestinal problems, and cancer.[18−22] For instance, the raw fresh young pieces of *Caralluma hexagona* (Syn; *Monolluma hexagona*, *Caralluma shadhbana*) are eaten as a cure for diabetes, abdominal pain, and stomach ulcers in traditional medicines in Yemen and Saudi Arabia.[23,24]

Following our previous phytochemical and pharmacological investigations of certain species of the genus *Caralluma*,[10,16,22,25−28] we report in this study the isolation and the detailed structure identification of four new pregnane glycosides from the CH_2Cl_2 fraction of the aerial parts of *Caralluma hexagona*. The enzyme-inhibitory activity of the total extracts, fractions, and isolated new compounds against glycoside and lipid hydrolases was tested *in vitro*. Also, at least the effects of the isolated compounds from the current and previous study (11 compounds, 2 of them as a mixture) on the formation of AGE products and their antioxidant activity were investigated.

2. RESULTS AND DISCUSSION

2.1. Identification of Pregnanes from *C. hexagona*. The CH_2Cl_2 fraction yielded seven known compounds.[10] Three were identified as pregnane glycosides for the first time (**4**, **5**, and **6**), two were known flavone glycosides [luteolin 4′-*O*-neohesperidoside (**7**)[29] and apigenin-8-*C*-neohesperoside (**8**)],[30] and two were known sterols [*β*-sitosterol (**9**)[31] and *β*-sitosterol-glucoside (**10**)][32] by comparison with similar standard samples (Co-TLC and IR) and spectral data in the literature. This fraction also afforded four new pregnane glycosides (**1**, **2**, **3A**, and **3B**) (Figure 1). A detailed identification of the new compound structures will be discussed below. Spectral data (^{1}H and ^{13}C NMR) of the new pregnane glycosides (**1**, **2**, **3A**, and **3B**) are included in the Supporting Information.

Compound **1** was isolated as a white amorphous powder (10 mg, $[\alpha]_{35}^{D}$ +1.3 in MeOH). The high-resolution electrospray ionization-mass spectrometry (HRESI-MS) analysis showed a molecular ion peak at 827.3623 $[M + Cl]^-$ (calcd. for $C_{41}H_{60}O_{15}Cl^-$, 827.3622) in negative mode, which was assigned to the molecular formula $C_{41}H_{60}O_{15}$, with 12 double-bond equivalents (DBE). The ^{13}C NMR spectrum of compound **1** showed 41 carbon signals. Five are corresponding to methyl carbons, 8 to methylene carbons, 21 to methine carbons, 1 to carbonyl carbon, and 6 to quaternary carbons. Twenty-one of the displayed carbons were assigned to the C_{21} steroidal part (Tables S1−S4). The NMR spectra of compound **1** showed great similarity with the spectra of compound **4**, which was previously isolated from *C. hexagona*.[10] The only difference is the lacking of the acyl group at C-20 in **1**, which is displayed by the upfield shift of C-20 (δ_C 65.1) relative to the same carbon in compound **4** (δ_C 74.1). This was further assured by comparing the data with those of similar pregnane glycosides.[33] Analysis of the NMR spectra of **1** showed the presence of two sugar moieties and one acyl group. The downfield shift of the protons and carbons at C-3 and C-12 confirmed that those were, respectively, the sites of glycosylation and acylation. This was confirmed from the results of the heteronuclear multiple bond correlation (HMBC) experiment (Figure S1). The sugar moieties were identified as *β*-D-glucopyranosyl-(1 → 4)-*β*-D-digitalopyranose at C-3 by comparison to the spectral data of compounds **4** and **5** with *β*-configuration of the anomeric protons (J = 7−9 Hz). The acyl group at C-12 was confirmed by referring to the published data of compound **4**. The ^{13}C peak values of the aglycone moiety of **1** (Tables S1−S4) were very similar to the data of compound **4** (3*β*,8*β*,12*β*,14*β*,20-pentahydroxy-(20*R*)-pregn-5-ene) except for C-16 and C-20. These differences predicted that the configuration at C-20 is different. To confirm this assumption, these data were compared with the ^{13}C peak values from literature for various 20*R*- and 20*S*-pregnane alcohols synthesized by the reduction of 20-

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pregnanons. These structures having hydroxyl moities at C-12 and C-14 resemble the boucerin or its 20*S*-epimer. Based on the results reported by Kimura et al.[34] and the ^{13}C chemical shift values of C-16 (δ_C 17.8) and C-20 (δ_C 65.1), the configuration of C-20 was determined as *S*-configuration. The comparison of the obtained data with those published by Al-Massarani et al.[17] confirmed the suggested configuration. Another evidence for the *S*-configuration came from the C-21 methyl group, which in 20*S* alcohols of C/D-cis pregnane-type steroids is close to the plane of the D-ring. It results in upfield shifts of the C-20 resonances (steric shift)[34,35] and the presence of correlation peaks between H-17/H-18 and H-18/H-20 in nuclear Overhauser effect spectroscopy (NOESY), which are but absent between H-18/H-20.[36] Therefore, compound **1** is the 20*S*-epimer of 3*β*,8*β*,12*β*,14*β*,20-pentahydroxy-(20*S*)-pregn-5-ene. Considering all of the abovementioned findings, compound **1** was identified as 12*β*-*O*-benzoyl 3*β*,8*β*,12*β*,14*β*,20-pentahydroxy-(20*S*)-pregn-5-ene-3-*O*-*β*-D-glucopyranosyl-(1 → 4)-*O*-*β*-D-digitaloside.

Compound **2** was isolated as an amorphous powder (18 mg): $[\alpha]_{35}^{D}$ +13.4 (*c*. 0.1, MeOH); HR-ESIMS *m*/*z* 941.4619 $[M + Na]^+$ in positive mode, also appearing in negative mode at *m*/*z* 953.4486 $[M + Cl]^-$ (calcd. for $C_{45}H_{74}O_{19}Cl^-$, 953.4512). From HR-ESIMS and ^{13}C NMR, **2** has the molecular formula $C_{45}H_{74}O_{19}$. The ^1H and ^{13}C NMR data of the aglycone and sugar moieties of **2** are presented in Tables S1−S4. The NMR data of the aglycone were similar to those of compounds **4** and **5**.[10] In comparison to **4**, compound **2** showed the disappearance of the hydroxyl moiety at C-12, confirmed from the presence of the upfield-shifted CH_2 group at δ_C 40.4 (C-12). Contrary to compound **5**, compound **2** showed an acylated sugar moiety at C-20. As for compound **1**, C-16 and C-20 appeared at ppm values of δ_C 18.7 and 77.9, respectively, clearly confirming an *S*-configuration at C-20. Therefore, the aglycone part of **2** was identified as 3*β*,8*β*,14*β*,20-tetrahydroxy-(20*S*)-pregn-5-ene. The analysis of ^1H and ^{13}C NMR spectra of **2** showed the presence of three sugar moieties attached to the aglycone with two sites that exhibit glycosylation and one acyl group attached to one of the sugar moieties. The sugar moieties were identified as two glucose and one digitalose (^1H and ^{13}C NMR spectra). The first glycosylation site was confirmed to be at C-3, similar to the sugar chains of **1**, **4**, and **5**, and the following glycosylation site was displayed at C-20. This was proved from the higher ppm values (+10 ppm) of the C-20 signal in relation to the data of nonsubstituted glycosides[36] and also from the observed long-range correlation between C-20 (δ_C 77.9) and H-1‴ (δ_H 4.39) of a glucose unit in the HMBC spectrum (Figure S2).

The presence of isovaleroyl moiety was confirmed from the ^1H NMR spectrum of **2**, which showed signals at δ_H 0.88, 2.20, and 2.06 due to two methyl, one methylene, and one methine groups, respectively.[27,37] The isovalerate group was suggested to be linked to the C-3‴ hydroxyl group of D-glucose moiety from the HMBC experiment. The HMBC correlations included a correlation between the signal assigned to a carbonyl carbon of the isovalerate group (δ_C 173.2) and H-3‴ (δ_H 4.86 Hz) of the sugar moiety, as well as correlations between the signal of proton H-3‴ (4.86 Hz) with both C-2‴ (δ_C 72.3) and C-4‴ (δ_C 68.2) and the anomeric carbon C-1‴ (δ 102.9). This was further confirmed from the upfield shift (−3 ppm) of the C-2‴ and C-4‴ signals compared to the data of nonsubstituted sugar.[38] It was not common that the C-21 steroidal glycosides contain an isovalerate group bound to a sugar moiety. The sugar chains were identified as *β*-D-glucopyranosyl-(1 → 4)-*β*-D-digitalopyranose at C-3 and 3‴-isoval-*β*-D-glucopyranoside at C-20 by comparison of the spectral data of **2** to the related glycosides.[27,37] Therefore, from the aforementioned data, compound **2** could be identified as 3*β*,8*β*,14*β*,20-tetrahydroxy-(20*S*)-pregn-5-ene-3-*O*-*β*-D-glucopyranosyl-(1 → 4)-*O*-*β*-D-digitaloside-20-*O*-3-Isoval-*β*-D-glucopyranoside.

Compounds **3A** and **3B** were obtained as a mixture (100 mg) as revealed from NMR data and mass spectrum (MS). This mixture could not be separated using high-performance liquid chromatography (HPLC) with different conditions. The single corresponding LC peak displayed a $[M + Na]^+$ at *m*/*z* 1045 for **3A** and at *m*/*z* 1065 for **3B**. The only difference comes from the acylating moieties, in which two benzoyl groups were identified in **3B**, and one isovalerate group and one benzoyl group in **3A**. A detailed study of their one-dimensional (1D)- and two-dimensional (2D)-NMR data helped the complete assignments of each compound of the mixture (Tables S1−S4). Therefore, these results suggested the presence of two compounds with a molecular formula of $C_{52}H_{78}O_{20}$ and $C_{54}H_{74}O_{20}$ for **3A** and **3B**, respectively. The spectra of the aglycone moieties of **3A** and **3B** were indistinguishable and showed great similarity with those of **2** except for the addition of an acyl group linked to the sugar moiety at C-20 in **3A** and **3B**. Compound **3A** showed the presence of two acyl groups: one identified as an isovalerate moiety located at C-3‴ [δ_C 75.4 and δ_H 5.22 (1H, m)] and one as a benzoyl moiety bonded to C-4‴ [δ_C 70.1 and δ_H 5.22 (1H, *t*, *J* = 9.2)]. The results were confirmed from the HMBC correlations (Figure S3), which showed correlations between the signal of carbonyl carbons (δ_C 165.2, 171.9) and H-3‴ and H-4‴′ of the D-glucose moiety, respectively. In **3B** (Figure 1), the isovalerate was replaced with the benzoyl group at C-3‴. The obtained ^{13}C-APT-NMR spectrum exhibited a similar aglycone part for **3A** and **3B** as shown by the overlapping pairs of signals with a ratio of about 2:1, respectively. Therefore, the aglycone part was identified as 3*β*,8*β*,14*β*,20-tetrahydroxy-(20*R*)-pregn-5-ene. The presence of a glucose moiety at C-20 benzoylated at positions C-3‴ and C-4‴′ was previously reported by Kunert et al.[39] The configuration at C-20 in **3A** and **3B** was identified as *R*-configuration in a similar way to compounds **4**−**6**,[10] based on the values of C-16 and C-20 and from the results of the rotating-frame Overhauser effect spectroscopy (ROESY) experiments (Figures S3 and S4). From the abovementioned data, the two compounds were identified as 3*β*,8*β*,14*β*,20-tetrahydroxy-(20*R*)-pregn-5-ene-3-*O*-*β*-D-glucopyranosyl-(1 → 4)-*O*-*β*-D-digitaloside-20-*O*-3-isoval-4-benzoyl-*β*-D-glucopyranoside (**3A**) and 3*β*,8*β*,14*β*,20-tetrahydroxy-(20*R*)-pregn-5-ene-3-*O*-*β*-D-glucopyranosyl-(1 → 4)-*O*-*β*-D-digitaloside-20-*O*-3,4 di-benzoyl-*β*-D-glucopyranoside (**3B**).

The hydrolysis of compounds **1**, **2**, **3A**, and **3B** using 1% HCl on a boiling water bath for 5 h each separately revealed the presence of two sugars, with one being much less polar compared to the other. Both sugars were identified in a similar way to our previous publication[10,36,37] as *β*-D-glucose by comparison with an authentic sample, in addition to the nonpolar sugar relative to glucose (6-deoxy sugar). The second sugar was identified as *β*-D-6-deoxy-3-*O*-methyl-D-galactopyranose (digitalose) by an extensive NMR study (1D- and 2D-NMR) and by comparison of its optical rotation values with those of the standard sugars.

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Table 1a. *In Vitro* Antioxidant Capacity of Total Extracts and Fractions from the Aerial Parts of *C. hexagona* Using DPPH, ABTS, and FRAP Assays[a]

	DPPH assay		ABTS assay	FRAP assay
extract/fraction	μM TE/mg extract	EC_{50} μg/mL	μM TE/mg extract	μM TE/mg extract
water extract	4.37 ± 13.51	>200	253.02 ± 14.24	32.17 ± 1.42
MeOH extract	39.37 ± 26.04	192.23 ± 1.65	199.86 ± 9.01	45.54 ± 6.27
methylene chloride fraction	60.03 ± 13.19	147.89 ± 4.91	242.75 ± 4.00	67.17 ± 4.15
MeOH/H_2O (1:1) diaion fraction (DCF-1)	128.53 ± 5.07	82.59 ± 3.38	378.58 ± 5.19	106.71 ± 5.66
MeOH diaion fraction (DCF-2)	94.03 ± 13.93	101.50 ± 4.21	317.75 ± 10.92	87.38 ± 14.29
ascorbic acid		2.76 ± 1.63		

[a]Data represent the mean ± SD.

Table 1b. *In Vitro* Antioxidant Activity of the Compounds Isolated from the Aerial Parts of *C. hexagona* Using DPPH, ABTS, and FRAP Assays

assay	DPPH assay	ABTS assay	FRAP assay
compound	TEAC (μM TE/mM)	TEAC (μM TE/mM)	TEAC (μM TE/mM)
1	43.30 ± 3.12	12.14 ± 14.86	17.96 ± 4.25
2	30.27 ± 2.70	9.19 ± 15.59	23.08 ± 1.89
3A, 3B	35.77 ± 11.52	9.42 ± 8.99	10.63 ± 2.74
4[a]	34.87 ± 4.89	7.08 ± 9.76	26.13 ± 4.80
5[a]	45.07 ± 4.80	27.36 ± 13.86	35.54 ± 7.39
6[a]	69.27 ± 11.19	148.64 ± 3.86	63.00 ± 0.94
β-sitosterol	37.87 ± 11.03	3.42 ± 9.77	13.50 ± 3.48
β-sitosterol-glucoside	36.27 ± 10.39	11.81 ± 7.07	66.21 ± 16.25
luteolin 4′-*O*-β-D-neohesperidoside	77.73 ± 8.95	599.19 ± 9.57	96.88 ± 8.99
apigenin-8-*C*-neohesperoside	168.80 ± 1.80	525.25 ± 5.82	278.21 ± 13.26
ascorbic acid	802.73 ± 32.63	1132.84 ± 20.25	1195.58 ± 4.41
quercetin	953.94 ± 4.17	1435.38 ± 14.02	1373.75 ± 35.39

[a]Pregnane glycosides were previously isolated by the authors.[10] Data represent the mean ± SD.

2.2. Antioxidant Activity. The antioxidant capacities of the total extracts, fractions of *C. hexagona*, as well as pure compounds obtained from CH_2Cl_2 fraction were evaluated (Tables 1a and 1b) using three different complementary methods (2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant potential (FRAP). The total antioxidant capacities for all tested samples were expressed as trolox equivalent antioxidant capacity (TEAC). The effective antioxidant activity could be determined by TEAC. There is a direct relation between TEAC and the antioxidant activity of the samples.

In the DPPH assay, the diaion column fractions DCF-1 (MeOH−H_2O, 1:1) and DCF-2 (MeOH) revealed the highest antioxidant capacities (128.53 ± 5.07 and 94.03 ± 13.93 μM trolox equiv/mg), respectively. Both fractions showed EC_{50} values of 82.59 ± 3.38 and 101.50 ± 4.21 μg/mL, respectively, compared to ascorbic acid (2.76 ± 1.63 μg/mL). The lowest radical scavenging activity was exhibited by the total aqueous extract (Table 1a). Among the isolated compounds, the antioxidant activity of the isolated flavonoidal compounds had higher potency (77.73 ± 8.95 and 168.80 ± 1.80 μM trolox equiv/mM) than the isolated steroidal compounds (30.27 ± 2.70 and 69.27 ± 11.19 μM trolox equiv/mM) compared to ascorbic acid and quercetin as positive controls (802.73 ± 32.63 and 953.94 ± 4.17 μM trolox equiv/mM, respectively) (Table 1b).

The results of ABTS assay revealed that the diaion fractions showed the highest antioxidant potency (378.58 ± 5.19 μM TE/mg for DCF-1 and 317.75 ± 10.92 μM TE/mg for DCF-2) due to their phenolic content. In this assay, the total aqueous extract showed greater antioxidant capacities than the total methanolic extract (Table 1a). Regarding the pure compounds, luteolin 4′-*O*-β-D-neohesperidoside showed the highest TEAC (599.19 ± 9.57 μM TE/mM) followed by apigenin-8-*C*-neohesperoside (525.25 ± 5.82 μM TE/m), while all other compounds (sterols and pregnane glycosides) showed very weak antioxidant capacities (Table 1b).

Regarding the FRAP assay, diaion fractions also showed higher antioxidant capacity than the other samples. Among the isolated compounds, apigenin-8-*C*-neohesperoside exhibited the highest antioxidant capacity (278.21 ± 13.26).

2.3. α-Glucosidase and Pancreatic Lipase Inhibition Activity. In our previous study,[10] we reported on the inhibitory capacity of the total extracts, fractions, and seven compounds isolated from the CH_2Cl_2 fraction against yeast α-glucosidase and pancreatic lipase enzymes. In a continuation of our interest in the bioactive CH_2Cl_2 fraction of *C. hexagona*, the new steroidal glycosides (**1**, **2**, and mixture of **3A** and **3B**) were tested for their α-glucosidase (yeast) and pancreatic lipase inhibitory activities (Table 2). The isolated compounds showed moderate α-glucosidase inhibitory activity with IC_{50} values of 0.92 ± 0.02, 0.67 ± 0.01, and 0.74 ± 0.02 μM, respectively, and lipase inhibitory activity with IC_{50} values of 5.17 ± 0.04, 18.20 ± 1.05, and 43.53 ± 2.02% at 100 μM, respectively. Several studies have been published on the yeast α-glucosidase inhibitory activity of pregnane glycosides.[19,40] In addition, the pregnane glycosides modulate food intake, improve lipid profile, or reduce intestinal fat absorption via inhibition of pancreatic lipase, which leads to a promising anti-obesity activity.[19,22,41] It is well known that acarbose is not very active against yeast α-glucosidase but mammalian enzymes.[42]

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Table 2. Inhibitory Effect of Pregnane Glycosides (1, 2, and 3A and 3B) Isolated from the CH_2Cl_2 Fraction of *C. hexagona* on α-Glucosidase and Lipase Enzymes[a]

assay	α-glucosidase assay	lipase assay
compounds/standards	IC_{50} (mM)	IC_{50} (μM)
1	0.92 ± 0.02	>100 μM
2	0.67 ± 0.01	>100 μM
3A and **3B**	0.74 ± 0.02	>100 μM
acarbose	0.81 ± 0.86	ND
orlistat	ND	7.41 ± 2.26

[a]ND; not determined. Data represent the mean ± SD.

Therefore, the effects of the MeOH extract and CH_2Cl_2 fraction of *C. hexagona* on glucose and insulin levels were studied *in vivo* in HFD/STZ-induced metabolic syndrome in rats (unpublished data). The results of the *in vivo* study further supported the results of enzyme-inhibitory activity.

2.4. Antiglycation Activity. The total aqueous, total methanolic extracts and methanolic fractions of *C. hexagona* as well as isolated compounds were evaluated for their scavenging activity of reactive carbonyls using three methods: BSA-fructose, BSA-methylglyoxal, and arginine-methylglyoxal. In the BSA-fructose model, the results showed that diaion fractions exhibited the best reactive carbonyl scavenging activity with an IC_{50} of 388.93 ± 20.95 and 567.97 ± 22.75 μg/mL for DCF-1 and DCF-2, respectively (Table 3a). This

Table 3a. *In Vitro* Antiglycation Activity of Total Extracts and Fractions from the Aerial Parts of *C. hexagona* Using Three Different Models[a]

	BSA-fructose model	BSA-methylglyoxal model	arginine-methylglyoxal model
extract/fraction	IC_{50} (mg/mL)	IC_{50} (mg/mL)	IC_{50} (mg/mL)
water extract	1.06 ± 0.09	>2	>2
MeOH extract	1.32 ± 0.06	>2	>2
methylene chloride fraction	1.93 ± 0.17	>2	ND
MeOH-H_2O (1:1) diaion fraction (DCF-1)	0.39 ± 0.02	>2	0.77 ± 0.10
MeOH diaion fraction (DCF-2)	0.57 ± 0.02	>2	1.49 ± 0.10

[a]Data represent the mean ± SD.

observation was consistent with the concentration of phenolic compounds in both fractions. The lowest activity was exhibited by the CH_2Cl_2 fraction (IC_{50} 1936.95 ± 171.1). Among the isolated compounds, apigenin-8-*C*-neohesperoside showed the highest antiglycation activity (IC_{50} = 75.39 ± 0.41 μM), even higher than quercetin (50.59 ± 3.90 μM) used as positive control (Table 3b). This result was in agreement with a previous study reporting a remarkable inhibitory activity of *C*-glycosyl flavones (vitexin and isovitexin) against the formation of AGEs induced by glucose or methylglyoxal, with efficacies of over 85% at 100 μM.[43] According to the literature search on *Caralluma*, no previous reports were found on the effects of the plants of the genus *Caralluma* on AGEs. Quercetin is an antioxidant drug used for treating many diseases; its antiglycation mechanism includes scavenging reactive oxygen species, trapping dicarbonyl compounds, and chelating metal ions.[44,45] The used BSA is an analogue of human serum

Table 3b. *In Vitro* Antiglycation Activity of Compounds Isolated from the Aerial Parts of *C. hexagona* Using Three Different Models[a]

compound	BSA-fructose model IC_{50} (μM)	BSA-methylglyoxal model IC_{50} (μM)	arginine-methylglyoxal model IC_{50} (μM)
1	502.36 ± 70.88	>1300	>1300
2	ND	>1300	NO
3A and **B**	452.67 ± 74.33	>1300	NO
4*	>1300	>1300	>1300
5*	>1300	>1300	>1300
6*	>1300	>1300	ND
β-sitosterol	>1300	>1300	>1300
β-sitosterol-glucoside	1032.44 ± 2.48	>1300	>1300
luteolin 4′-*O*-β-D-neohesperidoside	212.88 ± 0.66	70.73 ± 5.64	ND
apigenin-8-*C*-neohesperoside	75.39 ± 0.41	42.15 ± 3.75	40.24 ± 0.45
quercetin	50.59 ± 3.90	847.30 ± 1.04	726.90 ± 1.02

[a]NO; no inhibition; ND; not determined. *Pregnane glycosides were previously isolated by the authors.[10] Data represent the mean ± SD.

albumin because they have 90% homology.[46] Fructose is a highly reactive monosaccharide and forms glycation products that differ in both structure and reactivity as compared to those formed from glucose. Therefore, disease prevention through protein glycation inhibition mediated by fructose is highly recommended. This is because the synthesis of fructose was carried out through the oxidation of sorbitol in organs where the pathway of sorbitol is active, such as in the ocular lens and peripheral nerves.[47,48]

Antiglycation assay in the BSA-methylglyoxal model showed weak inhibition of fluorescent AGE production compared to the BAS-fructose model (Table 3a). Overall, the inhibition percentage of all extracts and fractions ranged from 15.50 ± 3.76 to 47.42 ± 2.84 at a concentration of 2 mg/mL. Among the isolated compounds, flavonoid compounds showed the highest antiglycation effect with IC_{50} values of 70.73 ± 5.64 and 42.15 ± 3.75 μM for luteolin 4′-*O*-β-D-neohesperidoside and apigenin-8-*C*-neohesperoside, respectively, compared to quercetin as a positive control (847.30 ± 1.04 μM) (Table 3b).

In the arginine-methylglyoxal model, diaion fractions also showed the most promising reactive carbonyl scavenging activity with an IC_{50} of 0.77 ± 0.10 and 1.49 ± 0.95 mg/mL for DCF-1 and DCF-2, respectively. On the other hand, luteolin 4′-*O*-β-D-neohesperidoside and apigenin-8-*C*-neohesperoside were significantly more active than quercetin (Table 3b). The reason for such differences was variations in the model systems. In the BSA-fructose model, both fructose and its oxidation products (such as methylglyoxal) react with protein to generate AGEs. The middle stage of protein glycation was evaluated by the BSA-methylglyoxal model as the conversion from sugars to carbonyls is omitted. Arginine is an important target for glycation of protein by reactive carbonyls. Methylglyoxal reacts with arginine through its guanidine group to form a fluorescent AGE called argpyrimidine.[6] Phytochemicals prevent AGE formation in part by functioning as free radical scavengers. Phenolic compounds exhibit a significant correlation between the free radical scavenger activity and the inhibitory effect on AGE generation.[49,50]

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3. EXPERIMENTAL SECTION

3.1. General Experimental Procedures. Optical rotations were measured on a JASCO P2100 polarimeter (Japan). Mass spectral data were carried out on a SHIMADZU LCMS-IT-TOF spectrometer. The NMR experiments for all of the isolated compounds were done using a Bruker AscendTM 400/R NMR spectrometer with the following frequencies: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz). Deuterated methanol (CD_3OD) was selected as the solvent of choice for the NMR experiments with TMS as internal standard, and chemical shifts were detected in δ ppm values.

3.2. Plant Material. Fresh aerial parts of *C. hexagona* Lavranos were obtained from Bani Habash Mountain, Almahweet, Yemen, in June 2017 and were chopped into pieces and dried at room temperature. The plant was authenticated by Dr. Abdul Wali A. Al Khulaidi, Associate Professor of Plant Geography, Flora and Vegetation, member of the Center of Middle East Plants, Al Baha University, Baljurashi, Saudi Arabia. Voucher samples (no. 10.7.2019.1) were kept at the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

3.3. Extraction and Isolation. The extracts and fractions were prepared according to the method previously reported by Shalabi et al.[10] The CH_2Cl_2 fraction (30 g) was chromatographed on a column of Si gel 60 (600 g, 63−200 μm, 5 × 100 cm^2) using CH_2Cl_2 and MeOH with an increasing polarity and MeOH (5−100%) as eluent. Fractions having the same pattern were collected together to yield eight fractions (**F1**−**F8**). Fraction **F5** (5.8 g) was chromatographed over a Si gel column (120 g, 3.5 × 50 cm) and eluted using CH_2Cl_2-MeOH (9.5:0.5, 9:10, 8.5:1.5 each 1 L) to give subfractions **F5-1** (0.3 g), **F5-2** (1.5 g), **F5-3** (0.7 g), **F5-4** (0.6 g), **F5-5** (0.42 g), **F5-6** (0.75 g), and **F5-7** (0.56 g). Fraction **F5-2** (0.55 g) was rechromatographed over an RP-18 Si gel column (30 g, 40−63 μm, 2.5 × 25 cm) using MeOH-H_2O (75:25 v/v) as the eluent to give three subfractions: **F5-1** (375 mg), **F5-2** (130 mg), and **F5-3** (45 mg). The subfraction **F5-1** was further purified on RP-18 silica gel column, yielding an inseparable mixture of **3A** and **3B** (100 mg). Fraction **F5-5** was purified on an RP-18 silica gel column to give compound **1** (10 mg) and compound **2** (18 mg).

3.3.1. Compound 1. Amorphous powder; $[\alpha]_{35}^{D}$ +1.3 (*c*. 0.1, MeOH); HR-ESIMS *m*/*z* 827.3623 $[M + Cl]^-$ (calcd. for $C_{41}H_{60}O_{15}Cl^-$, 827.3622) in negative mode. ^{1}H and ^{13}C NMR (400 MHz, 100 MHz, CD_3OD) data of aglycone and sugar moieties are presented in Tables S1−S4.

3.3.2. Compound 2. Amorphous powder; $[\alpha]_{35}^{D}$ +13.4 (*c*. 0.1, MeOH); HR-ESIMS *m*/*z* 941.4619 $[M + Na]^+$ in positive mode, also appears in negative mode at *m*/*z* 953.4486 $[M + Cl]^-$ (calcd. for $C_{45}H_{74}O_{19}Cl^-$, 953.4512). Tables S1−S4 show the ^{1}H and ^{13}C NMR (400 MHz, 100 MHz, CD_3OD) data of aglycone and sugar moieties.

3.3.3. Compounds 3A and 3B. White amorphous powder; the single corresponding LC peak displayed a $[M + Na]^+$ at *m*/*z* 1045 for **3A** and at *m*/*z* 1065 for **3B**; see Tables S1 and S2 for ^{1}H, ^{13}C NMR (400 MHz, 100 MHz, DMSO-d_6) data of aglycone moiety; and Tables S3 and S4 for ^{1}H, ^{13}C NMR data of sugar moieties.

Other compounds used in this study, namely **4**−**10** (Figure 1), were previously isolated from the plant as reported by Shalabi et al.[10]

3.4. Acid Hydrolysis. Each compound (**1**, **2**, and **3A** and **3B**) was subjected to acid hydrolysis based on the experiment carried out by Ahmad et al.[51] The aqueous layer of the reaction components was concentrated under vacuum and purified using a silica gel column according to according to Abdallah et al.[16] The column yielded two sugars, identified as D-glucose and 6-deoxy-3-*O*-methyl-D-galactose (digitalose) by comparison with the reference compounds on thin-layer chromatography (TLC) and by comparison of their optical rotation values. Digitalose was obtained by the hydrolysis of russelioside B, a major pregnane glycoside isolated by the author from *Caralluma russeliana*[36] and *Caralluma quadrangula*.[22]

3.5. *In Vitro* Assays. *3.5.1. Chemicals and Drugs.* All chemicals, solvents, enzymes, substrates, and standards are of high quality and analytical grade (see the Supporting Information).

3.5.2. In Vitro Antioxidant Activity. 3.5.2.1. DPPH Assay. The method depends on using and reducing the free radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH).[52] The procedure reported by Gutiérrez-Grijalva was followed on total extracts, fractions, or compounds using a 96-well plate. The positive controls selected were quercetin and ascorbic acid and the negative control used was MeOH. The experiment was carried out in triplicates for each sample (n = 3).

3.5.2.2. FRAP Assay. The method of ferric reducing antioxidant potential (FRAP) depends on reducing Fe^{3+} to Fe^{2+}, which is chelated by 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) to yield the Fe^{2+}-TPTZ complex.[53] An analytical curve was used to determine the antioxidant effect, using trolox (conc. 7.78−1000 μM) as standard (μM TE/mM). The positive controls selected are quercetin and ascorbic acid and the negative control, MeOH. The experiment was carried out in triplicates for each sample (n = 3).

3.5.2.3. ABTS Assay. The antioxidant capacity of the test samples to scavenge 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radicals was evaluated according to the method reported by Faitanin et al.[54] with slight modifications. The antioxidant activity was determined using an analytical curve, constructed with trolox (from 7.78 to 1000 μM) as standard (μM TE/mM). Ascorbic acid and quercetin were used as positive controls and MeOH as a negative control. Each sample was measured in triplicate (n = 3).

3.5.3. Enzyme-Inhibitory Assays. 3.5.3.1. Inhibition of α-Glucosidase. A colorimetric method with the substrate *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) was used to measure the α-glucosidase (yeast) inhibitory activity according to Gutiérrez-Grijalva[52] with slight modifications. Acarbose was used as a positive control (purity, 95%) and 5% dimethyl sulfoxide (DMSO) as a negative control. The following equation was used to calculate the percentage of inhibition of α-glucosidase

$$\%\ \text{inhibition} = [(\text{A blank} - \text{A sample})/\text{A blank}] \times 100$$

where A blank is the absorbance of the control (blank, without inhibitor), and A sample is the absorbance in the presence of the inhibitor.

The isolated compounds were presented at a final concentration of 1 mM to calculate the percentage inhibitory activity of α-glucosidase; then, serial dilutions were used to calculate the IC_{50}.

3.5.3.2. Inhibition of Pancreatic Lipase. The inhibition of pancreatic lipase activity was carried out using the substrate known as *p*-nitrophenyl palmitate (*p*-NPP) and porcine

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pancreatic lipase (PPL) described by Kordel et al.[55] with slight modifications. Orlistat was used as the positive control and 5% DMSO as the negative control or blank. The percentage of inhibition of lipase was calculated according to the equation

$$\% \text{ inhibition} = [(\text{A blank} - \text{A sample})/\text{A blank}] \times 100$$

The results were presented as a percentage of lipase inhibition for all compounds at 100 μM by comparing with orlistat as a standard. Absorbance was measured using a TECAN microplate reader (Tecan Co, Durham, USA).

3.5.4. Antiglycation Assays. 3.5.4.1. BSA-Fructose Assay. The inhibition test for the formation of advanced glycation end products (AGEs) using the BSA-fructose model was performed according to the method reported by Wang et al.[56] with slight modifications. The percentage of inhibition of AGEs was calculated according to the equation

$$\% \text{ inhibition} = [(\text{F blank} - \text{F sample})/\text{F blank}] \times 100$$

Percentage of AGE inhibition was presented at a final concentration of 2 mg/mL for the total extracts and fractions and at a final concentration of 1.3 mM for the isolated compounds; then, serial dilutions were prepared to calculate the IC_{50} for the most active samples.

3.5.4.2. BSA-Methylglyoxal Assay. The AGE inhibitory activity of the total extracts, fractions, or pure compounds was measured according to the method reported by Wang et al.[56] with slight modifications. The results were shown as glycation inhibition percentages, using the BSA-fructose model equation.

3.5.4.3. Arginine-Methylglyoxal Assay. The method used for the determination of the inhibitory effect of the total extracts, fractions, or pure compounds on the formation of advanced glycation end products in an arginine-methylglyoxal model was described by Wang et al.[56] with slight modifications. The results were shown as glycation inhibition percentages, using the BSA-fructose model equation.

4. CONCLUSIONS

Metabolic syndrome is characterized by some cardiometabolic risk factors that increase the risk of diabetes mellitus and cardiovascular disease, such as hyperglycemia, dyslipidemia, inflammation, abdominal obesity, coagulopathy, and hypertension. Reports have previously cited that *C. hexagona* extracts and the major constituents (pregnane glycosides and flavone glycosides) were beneficial against the metabolic syndrome through inhibition of glycoside and lipid hydrolases, as well as decrease of the oxidative stress and formation of AGEs. Despite much evidence from *in vitro* assays of improving these complications related to metabolic syndrome, further studies are needed to confirm the efficacy and safety of extracts/fractions or major compounds of *C. hexagona* in *in vivo* and clinical studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c02056.

Methods for bioassay and NMR detailed data; selected HMBC (→) and NOESY (↔) correlations of compound **1**; selected HMBC correlations of compound **2**; selected HMBC correlations of compound **3A**; and selected HMBC correlations of compound **3B** (PDF)

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Notes

The authors declare no competing financial interest.

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